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14. ABSTRACT

The first aim of this proposal is to identify small molecules that block NGAL function, a key molecule that our preliminary data suggested to be important in breast cancer and metastases. A high throughput screen was proposed to address this aim and currently we have constructed the essential reagents. The second goal is to examine the molecular mechanisms underlying NGAL function in breast tumorigenesis. In this regard, we have finished the mouse experiment depicting the role of NGAL in mammary tumor formation and the paper is in press in Cancer Research. In this publication, we found that ablation of mouse homologue of NGAL (mLCN2) significantly delayed the formation of mammary tumors in MMTV-ErbB2 (V664E) transgenic mice. We observed a much reduced tumor weight and delayed lung metastases in mLCN2 knockout background. Importantly, an affinity purified antibody against mLCN2 largely blocks the lung metastasis in the established 4T1 induced mammary tumors, suggesting the possibility of using a small molecule to inhibit NGAL function for breast cancer treatment.

15. SUBJECT TERMS
NGAL, NMP-9, breast cancer, metastasis, small molecule inhibitors

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Introduction

The first aim of this proposal is to identify small molecules that block NGAL function, a key molecule that our preliminary data suggested to be important in breast cancer and metastases. A high throughput screen was proposed to address this aim and currently we have constructed the essential reagents. The second goal is to examine the molecular mechanisms underlying NGAL function in breast tumorigenesis. In this regard, we have finished the mouse experiment depicting the role of NGAL in mammary tumor formation and the paper is in press in Cancer Research. In this publication, we found that ablation of mouse homologue of NGAL (mLCN2) significantly delayed the formation of mammary tumors in MMTV-ErbB2 (V664E) transgenic mice. We observed a much reduced tumor weight and delayed lung metastases in mLCN2 knockout background. Importantly, an affinity purified antibody against mLCN2 largely blocks the lung metastasis in the established 4T1 induced mammary tumors, suggesting the possibility of using a small molecule to inhibit NGAL function for breast cancer treatment.

Key research accomplishments

Aim1: to identify small molecules that block NGAL function and evaluate the effects of these new NGAL inhibitors in mouse breast cancer models (Yr 1-3).

We propose to use BiFC system (Bimolecular Fluorescence Complementation) to set up the high-throughput screen for small molecules that block NGAL function by disrupting the NGAL/MMP-9 complex formation. We have obtained the Venus system (similar to the original BiFC system but with much easier cloning sites) from Dr. Wichnick lab in University of Montreal. We have inserted NGAL into all four vectors in the system and tested its homodimerization in 293T cells (Figure 1). We also have constructed human MMP-9 in these vectors and the testing for the NGAL/MMP-9 complex formation is underway. As the Venus system is a transient transfection system, we have obtained Tet-regulated lentiviral vector (pTRIPz) from OPEN Biosystem. Once the formation of NGAL/MMP-9 complex is confirmed in the transient transfection experiment, we will clone the corresponding fragments into pTRIPz vector followed by virus production and establishment of stable cell lines for screening usage.

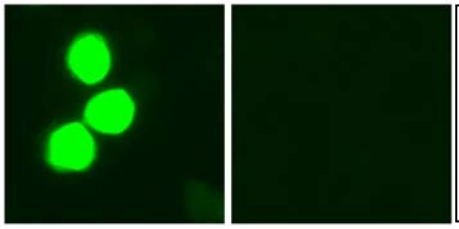

Figure 1: Testing the Venus system. (left) Co-transfection of NGAL(-SP)-Venus(1) and NGAL(-SP)-Venus (2) into 293T cells; homodimerized NGAL complexes resulted GFP signal. No GFP signals were detected in cells transfected with single plasmid alone (data not shown). (right) Untransfected 293T cells with the same exposure time.

Aim2: to examine the molecular mechanism underlying NGAL function in breast tumorigenesis.

Two essential tasks have been done in the first funding period. One is the preliminary data presented in the proposal have been finalized and the paper has been accepted to be published in Cancer Research (See Appendix for the paper).
Second, in the original proposal, we propose to dissect the gene expression profile using microarray to analyze the primary mammary tumor samples derived from the three mice groups: LCN2(+/+), LCN2 (-/+) and LCN2 (-/-). We mentioned that these samples were derived from two genetic backgrounds (C57/B6 and FVB/NJ) and the possible confusions might exist in our conclusions due to genetic heterogeneity. With this concern in mind, we have backcrossed the LCN2(-/-) mice (C57B) seven-times with FVB/NJ wild type mice and obtained mLCN2 knock-out mice in FVB/NJ background. We have already crossed these mice with MMTV-ErbB2 mice. The idea is to do microarray analysis on the tumors solely containing the FVB background to identify the changes in gene signatures that depend on LCN2 expression. Although this new approach will cause a bit delay, we think that it is definitely worth the waiting as the data shall be much more conclusive compared the original approach.

Reportable outcomes (Taken from in press paper in Cancer Research)

Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis

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Keywords: lipocalin 2, NGAL, breast cancer, metastasis

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Running title: Lipocalin 2 enhances breast cancer formation and metastasis

ABSTRACT
Lipocalin 2 (LCN2, also known as NGAL) is a secreted glycoprotein and its elevated expression has been observed in breast cancers. However, the importance of LCN2 in breast tumorigenesis is unclear. Here, we employed a spontaneous mammary tumor mouse model showing that MMTV-*ErbB2*(V664E) mice lacking of mLCN2 had significantly delayed mammary tumor formation and metastasis with reduced MMP-9 activity in the blood. LCN2 expression is up-regulated by HER2/PI-3 kinase/AKT/NF-κB pathway. Decreasing LCN2 expression significantly reduced the invasion and migration ability of $HER2^+$ breast cancer cells. Furthermore, injecting an anti-mLCN2 antibody into mice bearing established murine breast tumors resulted in significant blockage of lung metastasis. Our findings indicate that LCN2 is a critical factor in enhancing breast tumor formation and progression, possibly in part by stabilizing MMP-9. Our results suggest that inhibition of LCN2 function by an inhibitory

monoclonal antibody has potential for breast cancer therapy, in particular by interfering with metastasis in aggressive types of breast cancer.

INTRODUCTION

Recent studies have implicated LCN2, a member of the lipocalin family, in leukemia (1-3) and other solid tumors (4). The lipocalin family is a large group of small secreted glycoproteins involved in binding and transportation of small lipophilic molecules (5). The physiological ligand for LCN2 is not known. Upon bacterial infection, LCN2 tightly binds to bacterial iron-siderophores and deprives the iron source for their growth (6), which is shown by the delayed clearance of bacteria infection in LCN2 knock-out mice (7, 8).

It was observed that increased LCN2 levels were present in the plasma of leukemia patients with CML (1-3). We found that mouse LCN2 is required for BCR-ABL-induced leukemia in a mouse bone marrow transplant model and BCR-ABL^{+} mouse leukemic cells utilize the secreted LCN2 to reduce hematopoietic cells in normal bone marrow and spleen by inducing apoptosis of normal hematopoietic cells for leukemia cell expansion (1, 3). The requirement of LCN2 for leukemia cell expansion is consistent with the previously identified role of LCN2 in inducing apoptosis of mouse hematopoietic cells upon withdrawal of the cytokine growth factor IL-3 (9). The ability of LCN2 to mediate apoptosis appears to be due to its ability to export a still unknown iron-form from the target cells (10). Importantly, we found that LCN2 is also essential for solid tumor formation, as BCR-ABL-expressing leukemia cells lacking LCN2 expression failed to form subcutaneous allograft tumor nodules (3).

In breast cancer, a high molecular weight of complex consisting of LCN2 and MMP-9 was observed in the patient urine samples (11). Elevated LCN2 expression was shown to associate with decreased survival in breast cancer patients (12). Increased LCN2 expression was found to associate with increased tumor cell motility (13, 14). LCN2 was shown to stabilize MMP-9, a critical enzymatic activity required for extracellular matrix remodeling during tumor growth and metastasis (15, 16) and LCN2 may promote breast cancer progression by regulating epithelial to mesenchymal transition (14). However, the significance and how LCN2 is involved in breast carcinogenesis remain elusive.

We tested the role of LCN2 in breast tumorigenesis using a spontaneous MMTV-*ErbB2*(V664E) transgenic mammary tumor mouse model crossed with LCN2$^{-/-}$ mice. Our findings presented here illustrate the importance of LCN2 in breast cancer tumor formation and metastasis and the possible underlying molecular mechanisms promoted by LCN2 expression in breast cancer, indicating that inhibition of LCN2 has potential in breast cancer therapy.

Materials and Methods

Tissue culture and mice

Cell lines were grown in DMEM with 10% FBS and Pen/Strep at 37 ºC in humidified incubator with 5% CO_2. *LCN2* knockout (LCN2$^{-/-}$) mice were a gift from Dr. Alan Aderem (8). MMTV-*ErbB2*(V664E) transgenic mice (17) and athymic nude mice (8-12 wks) were purchased from The Jackson Laboratory and Harlan, respectively. Mice experiments were performed in MDACC animal facility with approved IACUC protocols.

Antibodies and constructs

Purified polyclonal antibodies against mouse or human LCN2 were generated in our lab using recombinant proteins purified from *E. coli*, a gift from Dr. Roland Strong (6). Antibodies against

AKT, pAKT, HER2, pY-HER2, and β-actin were purchased from Abcam. Dominant negative and constitutive active AKT constructs are gifts from Dr. Zhou Songyang.

shRNA experiments and lentiviral infection

shRNA lentiviral constructs (Sigma-Aldrich) containing shRNAs for *NGAL* (clone D: TRCN0000060290 and clone E: TRCN0000060290) and m*LCN2* (TRCN0000055328) were used. Puromycin was used to select cells containing shRNA after lentiviral infection.

RT-PCR

Total RNA extracted from cells was used to synthesize the cDNA as PCR template. RT-PCR primers for m*LCN2*: 5'-AGC CAG ACT TCC GGA GCG ATC-3'; and 5'-ACT TGG CAA AGC GGG TGA AAC G-3'; for mouse *actin*: 5'-GCT GGA AGG TGG ACA GTG AG-3' and 5'-ATG GAT GAC GAT ATC GCT GC-3'.

Two-chamber migration and invasion assays

Cell migration and invasion assays were performed using the two-chamber migration assay (8 μm pore size, BD Biosciences). For SKBr3 cells, 1 x 10^5 cells were seeded in serum-free medium in the upper chamber and migrated toward 10% FCS in the lower chamber for 18 hrs followed by fixation and staining with crystal violet 0.2%/methanol 20%. Quantification was performed using unpaired *t*-test.

Western blotting and zymogram of plasma samples

1-2 μl of plasma was separated on 20% SDS-PAGE followed by transferring and blotting with purified anti-mLCN2 polyclonal antibody (1:3000). 2-5 μl of plasma were analyzed using 10% zymographic gel (Invitrogen).

Imaging, histology, H&E staining and immunohistochemistry

Freshly collected lung tissues were imaged using XENOGEN IVIS 200 Imaging System for GFP signals. For histological analysis, tissues were post-fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E.

Statistical data analysis

The Kaplan-Meier method (the long-rank test) was used to determine the survival curves of tumor formation in MMTV-*ErbB2*(V664E) mice with various alleles of m*LCN2*. The log-rank test was used in the intergroup comparisons. The Wilcoxon rank-sum test was used to analyze the fluorescence imaging results of the in vivo antibody studies.

RESULTS

Up-regulation of LCN2 (NGAL) through HER2/neu signaling pathway in human breast cancer. We observed a significant clinical correlation of higher LCN2 expression with HER2 positivity among 318 newly diagnosed breast cancer patients (unpublished), implying that LCN2 might be a downstream target of the HER2 signaling pathway. When blocking HER2 function with Herceptin in $HER2^+$ SKBr3 cells, we found that LCN2 expression was inhibited (Figure 1A). LCN2 expression in SKBr3 cells was reduced in a dose-dependent manner by the PI-3K inhibitor LY294002 and by Bay 11-7082, which specifically blocks IκB phosphorylation needed for NF-κB activity (Figure 1B-C). Secretion of LCN2 into conditioned medium was also effectively inhibited under these conditions (Figure S1). In addition, overexpression of dominant negative AKT led to reduced expression of LCN2, while overexpression of constitutive active AKT increased LCN2 levels (Figure 1D). These results indicate a role of the HER2/PI-3K/AKT/NF-κB signal cascade in inducing LCN2 expression in human breast cancer, consistent with a recent study showing that LCN2 expression was largely dependent on the NF-κB pathway in thyroid neoplastic cells (18).

Mice lacking mLCN2 have impaired ErbB2(V664E)-induced mammary tumor formation. We tested mouse LCN2 (mLCN2) effects in a spontaneous mouse mammary tumor model. Transgenic mice carrying the mutant form of *ErbB2*(V664E) driven by the mammary specific promoter MMTV develop multiple primary breast tumors and lung metastases (17). By using MMTV-*ErbB2*(V664E)$^{tg/tg}$ mice (FVB) and m*LCN2*$^{-/-}$ mice (C57B/6) (8), we generated three groups of mice expressing ErbB2(V664E) with variations in m*LCN2* alleles (m*LCN2*$^{+/+}$, m*LCN2*$^{+/-}$, and m*LCN2*$^{-/-}$) (Figure S2A-C).

In this genetic study for the effects of mLCN2 on *ErbB2*(V664E)-induced breast tumors, we observed striking differences in the timing of tumor formation, as well as the number and the size of primary tumors among the three groups. Groups carrying either one or two alleles of m*LCN2* started to develop multiple large (>1 cm) mammary tumors around 170 days after birth. In contrast, m*LCN2*$^{-/-}$ mice did not form similar size of tumors until approximately 260 days, a time when >60% mice in the groups expressing mLCN2 were already terminated due to excessive tumor burden (Figure 2A). The overall tumor occurrence was significantly delayed in the m*LCN2*$^{-/-}$ group (260 – 500 days with T_{50} = 303 days) compared to the m*LCN2*$^{+/+}$ mice (170-340 days with T_{50} = 210 days) (Figure 2A). Although the m*LCN2*$^{+/-}$ group showed a slightly delayed course of tumor occurrence compared to the m*LCN2*$^{+/+}$ group, no significance was observed regarding the tumor occurrence and the tumor volume between these two groups (Figure 2A-B), indicating that one allele m*LCN2* deficiency was not sufficient to interfere with the formation of ErbB2(V644E)-induced breast tumors. We observed greater numbers and larger volumes of tumors per mouse in the groups expressing mLCN2 compared to the m*LCN2*$^{-/-}$ group (Figure 2B and Figure S2D). Notably, the lung metastases in m*LCN2*$^{-/-}$ mice were significantly delayed compared to the m*LCN2*$^{+/+}$ mice ($p < 0.05$) (Figure 2C). By Kaplan-Meier analysis, the T_{50} for lung metastasis in the m*LCN2*$^{+/+}$ group is approximately 260 days. In contrast, the T_{50} value was not reached in the m*LCN2*$^{+/-}$ and the m*LCN2*$^{-/-}$ groups, suggesting that deficient m*LCN2* expression also impairs lung metastasis in this model.

Unlike the healthy WT mice with minimal levels of mLCN2 in the plasma (Figure 2D, top panel, last three lanes), we detected a dramatically increased mLCN2 level in the plasma of tumor-bearing MMTV-*ErbB2*(V664E) mice (Figure 2D, top panel). Interestingly, we observed elevated MMP-9 gelatinase activity and the presence of higher molecular weight gelatinase

(HMW MMP) activity in the plasma of tumor-bearing mice expressing mLCN2 compared to m*LCN2*$^{-/-}$ group (Figure 2D, bottom panel, outlined with red box). We also noticed the relatively weak MMP-9 gelatinase activity in the plasma of tumor-bearing mLCN2$^{-/-}$ mice (Figure 2D, bottom panel), and these samples displayed distinct gelatinase bands with MW lower than the MMP-9 band, suggesting the beneficial effects of mLCN2 in maintaining MMP-9 activity potentially though stabilizing MMP-9, as implied by the previous studies (15, 16)**.**

LCN2 expression in HER2^{+} breast tumor cells stimulates cell invasion *in vitro* and their metastatic potential in mouse xenograft model. Using HER2^{+} SKBr3 cells (high LCN2 expression), we observed a significant reduction in cell migration and invasion upon knocking down LCN2 expression compared to either parental cells or cells expressing non-specific shRNA (Figure 3A-B). We used MDA-MB-468 (high LCN2 expression) to test LCN2 effects in mouse xenograft model. Note, we did not observe any change in cell growth rate in culture upon lowering LCN2 expression by shRNA in either SKBr3 or MDA-MB-468 cells (data not shown and Figure S3A). We injected mammary fat pads of nude mice with one million of either parental MDA-MB-468 cells, or its derivatives expressing either the non-targeted shRNA or the shRNA for LCN2. We analyzed the primary tumor and the surrounding tissues 42 days after implantation. No significant differences in the primary tumor size/weight were found among the three groups (Figure S3B). However, the tumor cells' capacity for invasion and metastasis, as measured by the events of lymphovascular invasion (Figure 3C, panel b), intramammary lymph node metastasis (Figure 3C, panel c) and chest/abdominal wall invasion (Figure 3C, panel d) were significantly reduced in the group injected of MDA-MB-468 cells with the *LCN2* shRNA knockdown (Figure 3D).

mLCN2 expression correlates with aggressive tumor formation in murine mammary tumor cell lines. The association of LCN2 expression with aggressive human breast cancer types was further demonstrated when using a series of mouse breast tumor cell lines (67NR, 168FARN, 4T07, and 4T1), which were derived from the mammary tumors of the same mouse but with distinct metastatic potentials (19). 4T07 and 4T1 cells are the most aggressive and develop lung metastases. We found that only 4T07 and 4T1 cells have m*LCN2* transcripts and secrete mLCN2, with the most aggressive 4T1 cells having the highest levels of mLCN2 (Figure 4A). Knocking down m*LCN2* by shRNA in 4T1 cells (Figure S4A, left panel) reduced MMP-9 activity (Figure S4A, right panel). Reduction of mLCN2 expression greatly decreased 4T1 invasive ability (Figure S4B) and colony formation in soft agar (Figure S4C). Similar to our findings in the plasma from *ErbB2*-induced breast tumor-bearing mice, we detected high levels of mLCN2 in the plasma of breast tumor-bearing mice implanted with 4T1 cells and increased MMP-9 activity in the plasma compared to normal healthy mice (Figure S4D).

Intravenous administration of anti-mLCN2 antibody to mice with established mammary tumors reduces lung metastases. To explore the possibility that inhibition of the secreted LCN2 might block distant metastasis, we intravenously injected an affinity-purified rabbit polyclonal antibody against mLCN2 (anti-mLCN2) into nude mice seven days after implantation with 5000 GFP-labeled 4T1 cells, when visible breast tumors (~2 mm) were formed. Antibody injection (~100 μg) was done once per week for four times, with purified rabbit IgG as control. We observed a dramatic decrease in lung metastases in the anti-mLCN2 antibody treated group as compared to the control IgG-treated group, measured by GFP signal intensities in freshly

collected lung tissues (p=0.028) (Figure 4B-C and Figure S5A). Histological examination confirmed significant differences in lung metastases between the two groups (Figure 4D). We also found that the levels of circulating 4T1 tumor cells were reduced in the blood of anti-mLCN2 antibody-treated mice (Figure S5B), suggesting that the antibody was able to block the emergence of disseminating tumor cells from the primary tumor sites. Taken together, our results strongly suggest that LCN2 could serve as a new therapeutic target for treating breast cancer metastasis.

DISCUSSION

The results presented here provide strong pre-clinical and experimental evidence that LCN2 is a critical factor that facilitates breast tumorigenesis and metastasis, and it is a potential therapeutic target for aggressive forms of breast cancer. We were the first to use a spontaneous mouse breast cancer model to study the function of LCN2 in breast tumorigenesis. Mice that lack mLCN2 expression had a significant delay in the formation of breast tumors in the MMTV-*ErbB2*(V664E)^{tg} background, and when formed, these tumor displayed reduced tumor numbers and size compared to that from the m*LCN2*$^{+/+}$ and m*LCN2*$^{+/-}$ mice. The timing of lung metastases was correspondingly delayed in the m*LCN2*$^{-/-}$ mice compared to the m*LCN2*$^{+/+}$ mice. This result indicates that although breast tumor formation and metastasis may not absolutely require LCN2 expression, LCN2 plays an essential role in enhancing the aggressiveness of breast cancer formation and metastasis. Our studies using human breast cancer cell lines and murine mammary tumor cells indicate that LCN2 expression led to more aggressive behavior including lung metastasis, tumor cell migration and invasion, and anchorage independent growth *in vitro*.

How LCN2 is involved in solid tumor formation and progression is not fully understood. We observed an overall increased MMP-9 gelatinase activity, formation of higher molecular weight MMP complexes (20), and increased MMP-9 stability in tumor-bearing mice expressing LCN2. In contrast, in tumor-bearing mice with ablated LCN2 expression, an overall decrease in higher MW MMP activities and MMP-9 activity was observed , consistent with the previous findings of the protective role of LCN2 on MMP-9 (16). The importance of these higher MMP activities in tumorigenesis is not known. The effects of loss of LCN2 expression on these MMP-related events are not yet understood and need further investigation.

Most importantly, we provided evidence that i.v. injection of an affinity-purified antibody made against mLCN2 strongly interfered with lung metastasis in an aggressive mouse 4T1-induced mammary tumor model. The primary breast tumors were not prevented from continued growth by i.v. injection of the mLCN2 antibody, which may be due to the insufficient levels of the mLCN2 antibody in reaching an already-established tumor microenvironment. However, we did observe that anti-mLCN2 antibody-treated mice had slightly smaller primary tumors (10% reduction on average, data not shown). We found less 4T1 cells in the blood of anti-mLCN2 antibody-treated mice. Possible mechanisms include that the anti-mLCN2 destabilizes LCN2/MMP-9 complex, there by reducing the exit of 4T1 cells from the primary tumor mass and decreasing distant tumor metastasis. More studies are needed to clarify the mechanism of how anti-LCN2 reduces metastases. Our findings suggest that blocking LCN2 in blood circulation using a neutralizing monoclonal antibody against LCN2 might be an effective therapeutic option to interfere with breast cancer metastasis either alone or in combination with current therapeutic approaches.

Figure

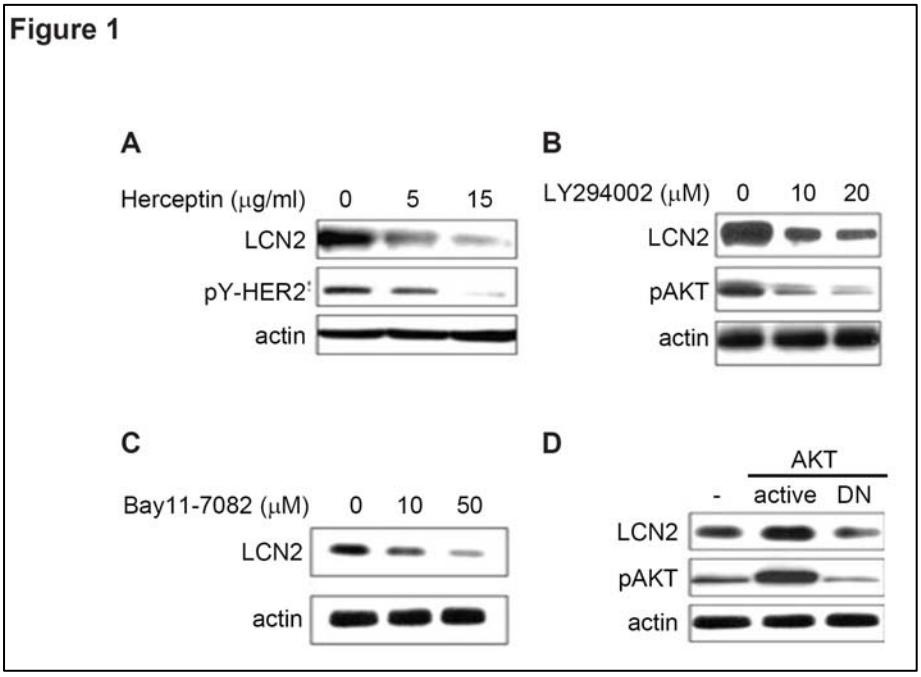

Figure 1. LCN2 (NGAL) is a down-stream target of HER2/PI-3K/AKT/NF-κB pathway. *A*, Western blotting of LCN2 levels in $HER2^+$ SKBr3 cells treated with Herceptin. *B*, Western blotting of LCN2 levels in SKBr3 cells incubated with the PI-3K inhibitor LY294002. *C*, Western blotting of LCN2 levels in SKBr3 cells incubated with the NF-κB inhibitor Bay 11-7082. *D*, Western blotting of LCN2 levels in SKBr3 cells transiently expressing either constitutive active or dominant negative AKT.

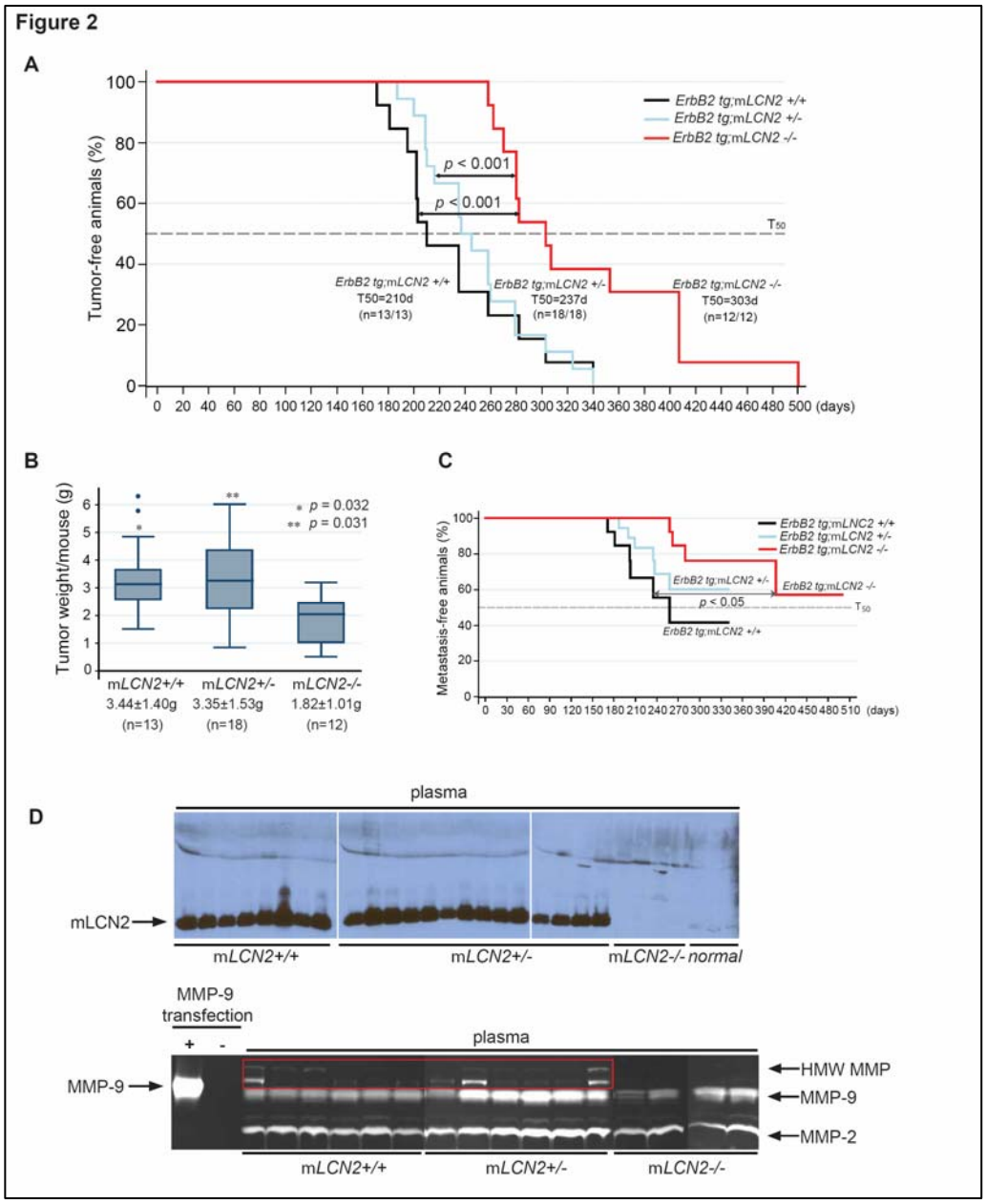

Figure 2. Effects of mLCN2 on the mammary tumor formation and metastasis in the MMTV-*ErbB2*(V664E) transgenic mouse model. *A*, time course of primary mammary tumor development in MMTV-*ErbB2*(V664E) transgenic mice in the three genetic backgrounds: m*LCN2*$^{+/+}$, m*LCN2*$^{+/-}$, and m*LCN2*$^{-/-}$, depicted by the Kaplan-Meier analysis. T_{50} is a calculated statistical value incorporating both time and incidence of tumor formation when 50% of the mice in the same group developed mammary tumors. *B*, summary of total breast tumor volumes per mouse in the three mice groups. *C*, Kaplan-Meier analysis of lung metastasis of the three mice groups. *D*, levels of mLCN2 (upper panel) in the plasma of the three mice groups at euthanization. mLCN2 level in the plasma from normal healthy mice was shown in the last three lanes. MMP activity in the plasma of the three mice groups were analyzed using 10% zymogram gel (lower panel). Conditioned medium from MMP-9-transfected 293T cells were used to mark MMP-9 position. Red box outlined the high molecular weight of MMP activity (HMW MMP).

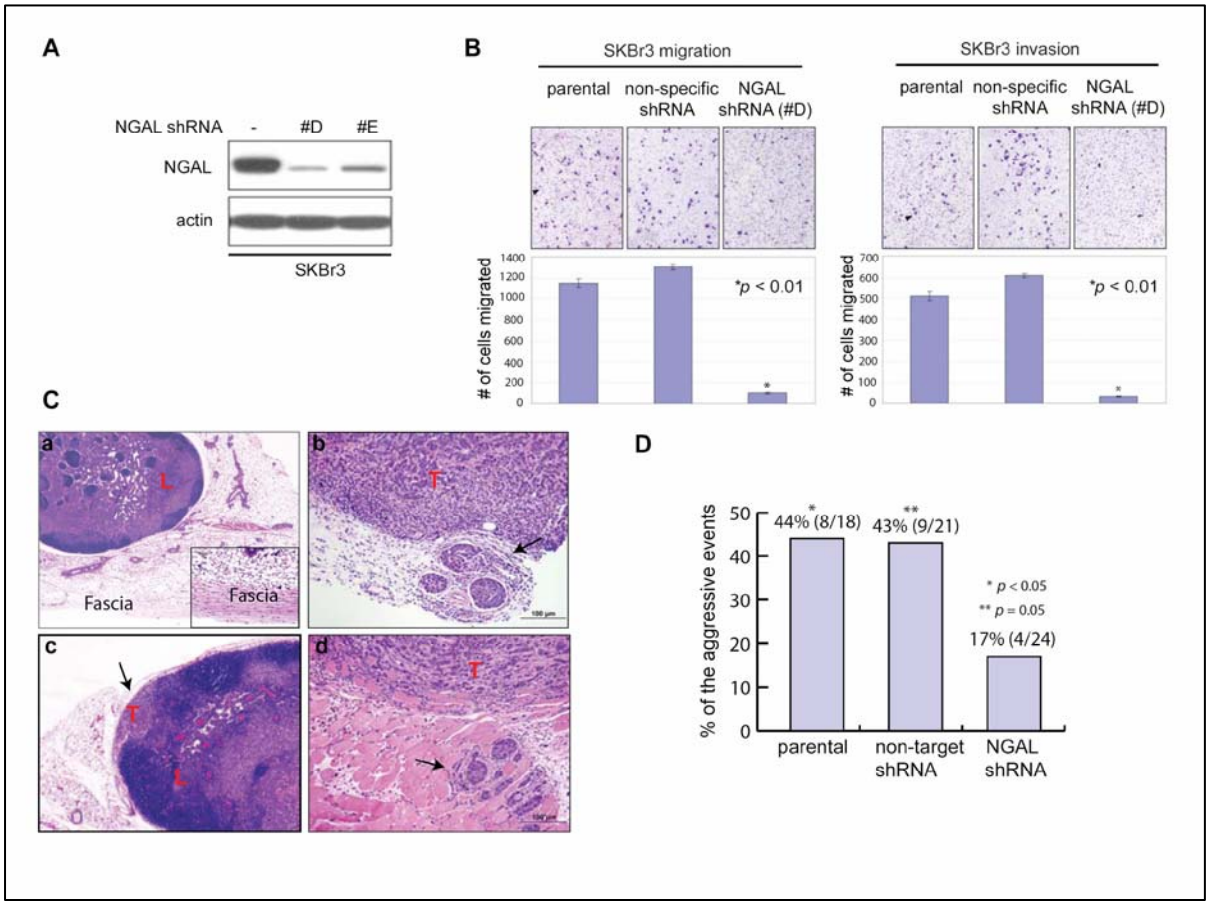

Figure 3. LCN2 (NGAL) expression stimulates cell invasion and metastasis of human breast cancer cells. *A,* Western blotting of NGAL levels in SKBr3 with shRNA targeting NGAL. *B,* cell migration (left panel) and invasion (right panel) assays of SKBr3 cells, SKBr3 cells expressing non-specific shRNA, or SKBr3 cells expressing *NGAL* shRNA. *C,* H&E staining of the 4th mammary gland from normal mouse (a) showing normal intramammary lymph node structure and fascia as a barrier to block tumor cells from invading into the chest/abdominal wall. Pathological features (black arrow) including lymphovascular invasion (b), intramammary lymph node metastasis (c) and chest/abdominal wall invasion (d) were used to measure the metastatic and locally aggressive events in the whole mount mammary fat pad in the experiment performed in (D). "L" represents lymph node; "T" represents tumor. *D,* summary of the lymph node metastasis and locally aggressive events observed in the mammary glands injected with MDA-MB-468 cells, or MDA-MB-468 cells expressing either non-targeted shRNA or *NGAL* shRNA. Statistical analysis was performed with Pearson Chi Square test.

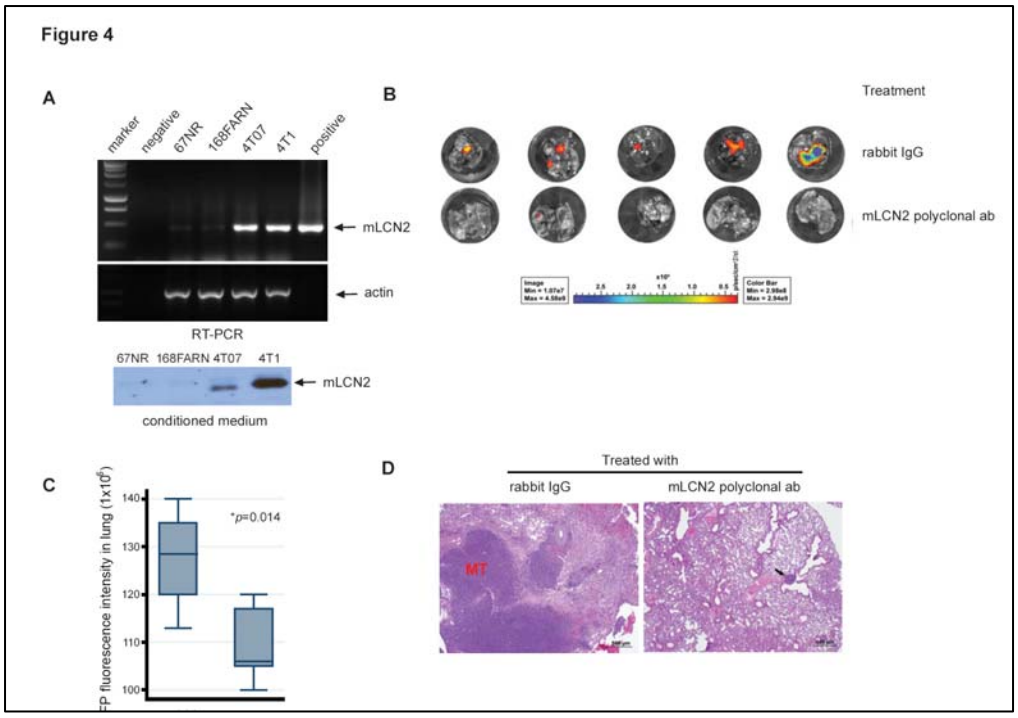

Figure 4. Reduction of lung metastasis by mLCN2 antibody injection in the 4T1 breast cancer model. *A*, expression of mLCN2 is associated with aggressive mouse mammary tumor cell lines. Upper panel: RT-PCR of mLCN2 transcripts in 67NR, 168FARN, 4T07 and 4T1 cells; actin as internal control and a plasmid containing mLCN2 as positive control. Lower panel: Western blotting of mLCN2 levels in the conditioned medium of the four cell lines. *B, in vivo* metastasis assay of 4T1 cells of mice injected with purified rabbit IgG (upper panel) or purified rabbit anti-mLCN2 polyclonal antibody (lower panel). Lung metastasis burden was monitored using Xenogen IVIS 200 Imaging System. The color scale depicts the GFP signal emitted from the metastatic 4T1 cells. *C*, comparison of total GFP signal intensity in either the control antibody-treated group or the anti-mLCN2 antibody-treated group. *D*, representative H&E staining of lung metastasis of 4T1 tumor bearing mice treated with purified rabbit IgG (left panel) or purified anti-mLCN2 antibody (right panel); "MT" represents metastatic tumor. Black arrow points to a small metastatic tumor lesion.

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Conclusions

Published studies and our unpublished clinical data indicate the association of elevated LCN2 expression with HER2-positive breast cancer patients, but its role in breast cancer is not known. Here, we used a mouse mammary tumor model to elucidate the role of LCN2 in breast tumorigenesis. We found that LCN2 knockout mice have a significant delay in mammary tumor formation and metastasis in MMTV-ErbB2(V664E)-transgenic background. Importantly, an affinity purified polyclonal antibody against mouse LCN2 largely blocks lung metastasis in mice with established 4T1 mammary tumors, suggesting that inhibitory molecules against human LCN2 will have medical utility for breast cancer treatment.

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Publications

Leng XH, Ding T, Lin H, Wang Y, Hu L, Hu J, Feig B, Zhang W, Pusztai L, Symmans WF, Wu Y, Arlighaus RB. **Inhibition of Lipocalin 2 Impairs Breast Tumorigenesis and Metastasis.** *Cancer Research*, in press.

Leng X, Ding T, Wu Y, Arlinghaus R. **Requirement of lipocalin 2 in breast cancer.** Adv Enzyme, 2010 (invited article).

Meetings

Poster
100 AACR annual meeting, April 18-22, 2009, Denver, CO

Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis

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Keywords: lipocalin 2, NGAL, breast cancer, metastasis

Abstract
Despite of the detection of higher molecular weight complex containing MMP9 and human lipocalin 2 (LCN2, also NGAL) in the urine samples of breast cancer patients, the clinical significance and the biological mechanism underlying this observation are not yet known. In this study, we analyzed LCN2 expression in more than 300 samples of breast cancer cases using molecular genetic profiling and found that elevated LCN2 expression is associated with aggressive and advanced types of breast cancers such as ER negative, HER2 positive, advanced disease stages, high nuclear grade and positive for node metastasis. More importantly, we have validated the role of LCN2 in breast tumorgenesis using a mouse genetic model and other experimental approaches. By crossing LCN2(-/-) mice with MMTV-HER2(V664E)^{tg} mice, we found that HER2(V664E)^{tg} mice without LCN2 allele showed a significant delay in primary breast tumor formation and a much reduced tumor volume compared to HER2(V664E)^{tg} mice with LCN2 allele(s). In addition, MMTV-HER2(V664E)^{tg};LCN2(-/-) mice displayed a great reduction in lung metastasis compared to HER2(V664E)^{tg} mice with LCN2 allele(s). We found that HER2-induced LCN2 expression is through PI3K/AKT/NF_kB pathway. Modulation of LCN2 expression in established human breast cancer cell lines has profound effects on cell motility and invasive ability. Ectopic expression of LCN2 in non-tumorgenic human breast

cancer cell ZR75.1 induced tumor formation in a xenograft model, whereas reducing LCN2 expression in tumorgenic human breast cancer cell MDA-MB468 greatly blocked its invasion activity in vitro and in vivo. Using a murine breast cancer cell line (4T1), we found that i.v. injection of antibody against LCN2 largely blocked lung metastasis. Our results indicated that expression and secretion of LCN2 is an essential player in breast cancer tumor formation and metastasis. Inhibition of its function using such as an antibody against LCN2 could be a feasible approach for designing new cancer therapy.

Presentation
50th Advances in Enzyme Regulation Symposium, Bologna September 27-29.
Abstract

Mechanistic knowledge of molecular markers (e.g. HER2) has increased our understanding of breast tumorigenesis, and has lead to effective treatments and diagnoses. To determine its role in breast cancer, we analyzed human LCN2 (NGAL) expression using data derived from 318 primary breast cancer samples and found increased NGAL expression is associated with certain aggressive forms of breast cancer. We also analyzed a series of human breast cancer cell lines, and identified high LCN2 expression in HER2 and epidermal growth factor receptor positive cell lines (such as SKBr3 and MDA-MB-468) and low expression in estrogen receptor (ER) positive cell lines (such as ZR75.1 and MCF-7). Using both the human and mouse mammary tumor cell lines, we found that elevated LCN2 expression increased cell migration and invasion both in cell culture and in mouse tumor studies. The elevated LCN2 expression is up regulated by HER2/PI-3 kinase/AKT/NF-kB pathways. To further characterize the role of LCN2 in breast cancer formation and progression, we established a mouse genetic model. We demonstrated that mice lacking LCN2 were significantly impaired for induction of ErbB2-induced breast tumor formation. Importantly, injection of affinity-purified antibody against 24p3 in mice with established 4T1-induced breast tumors significantly reduced lung metastasis. Taken together, our finds illustrated the molecular mechanism involved in breast cancer progression promoted by LCN2, and indicate that inhibition of LCN2 is a potential new therapeutic approach for treating human breast cancer, in particular, by blocking metastasis of aggressive types of breast cancers.

List of Personnel (not salaries) receiving pay from the research effort

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Arlinghaus, Ralph B.
Wang, Yang

Appendices

Email about the paper acceptance in Cancer Research

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